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DIFFERENTIATION OF THE EPIDERMIS OF NECK, TAIL AND LIMBS IN THE EMBRYO OF THE TURTLE *EMYDURA MACQUARII* (GRAY, 1830)

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Abstract. The development of the skin in turtles and the differentiation of the first keratinized layers are largely unknown processes. The histology and ultrastructure of the developing skin of neck, tail and limbs of the embryo of the turtle *Emydura macquarii* were studied. This study showed that three to four embryonic layers are initially formed from the basal layer. They contain scarce bundles of 8-12 nm-thick intermediate filaments of keratin, and many coarse 28-35 nm-thick filaments of unknown nature. The coarse filaments form reticulate bodies similar to those of lepidosaurian reptiles and birds, and form large aggregations within corneocytes of the embryonic epidermis. Embryonic layers also contain mucus and vesicular bodies, the latter associated with lipid droplets. Lipids and mucus are partly discharged into the amniotic fluid. Shortly before hatching, typical α-keratinocytes, containing keratin filaments and no coarse filaments, replace the embryonic epidermis. Mucus and lipids are, however, retained among α-keratinocytes after hatching. The loose dermis of early embryonic stages rapidly turns into a dense connective tissue that strengthens the delicate epidermis. Large collagen fibrils contact the basement membrane of the epidermis of the tail. Lipidic material is also stored in dermal fibroblasts.

Key words : turtle (*Emydura macquarii*), embryo, soft skin, ultrastructure.

INTRODUCTION

Chelonia are considered among the most ancient fully terrestrial vertebrates (McFARLAND *et al*. 1979). The skin of these reptiles is adapted to protect the body from desiccation and mechanical stress on land. The epidermis varies in histological and biochemical composition according to the body regions.

In the shell (carapace and plastron), a hard and variably thick layer of horny cells is composed of β- (ϕ)-keratins (SPEARMAN, 1966 ; ALEXANDER, 1970, PARAKKAL & ALEXANDER, 1972 ; BADEN *et al.*, 1974 ; WYLD & BRUSH 1979, 1983). This hard type of keratin shows a typical X-ray diffraction pattern, a fibril periodicity of 2-3 nm, and barely stains with toluidine blue, eosine or other dyes. β-keratinocytes merge completely or partially with one another to form a syncytial β-keratin layer (ALEXANDER, 1970 ; ALEXANDER & PARAKKAL, 1969 ; LANDMANN, 1979 ; 1986 ; MADERSON, 1985 ; MADERSON *et al.*, 1998).

The specialized epidermis of the shell derives from embryonic regions where peculiar dermo-epithelial interactions take place (RUCKES, 1929; EWERT, 1985; BURKE, 1989a,b, 91). This epidermis forms placodes under which dermal cells aggregate.

In the embryonic skin of the head, neck, limbs and tail, similar dermo-epidermal interactions are not present, and the adult skin of these areas is soft, pliable and capable of folding. The epidermis of these regions is covered by α-keratinocytes (ALEXANDER, 1970; SPEARMAN, 1969; PARAKKAL & ALEXANDER, 1972; MATOLTSY & HUSZAR, 1972). Contrary to β-keratin, the soft α-keratin presents its specific X-ray diffraction pattern, a fibril periodicity of 8-12 nm, similar to that of mammalian keratins (BADEN & MADERSON, 1970). Also, mature α-keratinocytes are very thin (0.2-1.0 μm), do not merge with one another but remain separate, and are stainable with toluidine blue, eosin and other dyes.

Contrary to most turtles (adapted to a freshwater environment), in some tortoises (adapted to a more terrestrial and dry environment) also the outer part of numerous limb and neck scales contains hard β-keratin, which alternates with softer α-keratin on the inner side and hinge regions (SPEARMAN, 1966, 1969; BADEN & MADERSON, 1970). These large and tough scales enhance the mechanical and defensive protection when the animal retracts the limbs into the shell.

Although the process of keratinization has been studied histologically, histochemically and ultrastructurally in the soft skin regions of adults (SPEARMAN, 1966, 1969; HENRIKSON & MATOLTSY, 1970; MATOLTSY & HUSZAR, 1972; MATOLTSY & BEDNARZ, 1975; MATOLTSY, 1987), to date there are no reports on the modality of keratinization during skin embryogenesis up to the time of emergence of the hatchling.

The present ultrastructural study, together with others on the shell morphogenesis and on the differentiation of the skin in the carapace and plastron (ALIBARDI & THOMPSON, 1999a,b), describes the modifications of the epidermis during the passage from the liquid environment of the embryo, surrounded by the amniotic fluid, to the dry and freshwater environment of the adult.

MATERIAL AND METHODS

Eggs of the Australian short-necked pond turtle *Emydura macquarii*, Gray 1830, collected during summer in the countryside of New South Wales, were used in this study.

The tables of development by YNTEMA (1968) on the freshwater turtle *Chelydra serpentina*, were used as a reference system. Collected embryos were representative of embryonic stages (ES) 15 (n=3), ES 16 (n=2), ES 18 (n=1), ES 19 (n= 3), ES 22 (n=1), ES 23 (n=3), ES 24 (n=7), ES 25 (n=3), 1-2 post-hatching (n=2), 1 week post-hatching (n=2).

Pieces (1-5 mm large) of embryonic tissues were fixed in cold (0-4°C) 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. After rinsing in the same buffer, the tissues were post-fixed in 1% osmium tetroxide for about 2 h (some pieces also placed in 4% uranyl acetate for 1-2 h), dehydrated and embedded in Durcupan or Spurr resins.

The skin of limbs, neck and tail, was sectioned with an ultramicrotome in cross or sagittal planes in order to collect representative areas. One to 2 μm-thick semithin sections

were stained with 0.5% toluidine blue or toluidine blue-eosine (5-10 seconds each on a hot plate). Thin, 50-90 nm-thick, sections were collected on copper or nickel grids, routinely stained with uranyl acetate and lead citrate, and observed in a transmission electron microscope Philips CM 100, operating at 60-80 kV.

RESULTS

Light microscopy

At ES 15-16, most of the epidermis covering the studied regions was bilayered, and few suprabasal cells were seen. Mitotic cells were frequently observed in the epidermis and in the outer flat peridermis (Fig. 1). Between ES 17 and ES 22, 1-2 layers of suprabasal cells were produced, which did not accumulate keratin but were rich in pale vesicles resembling lipid droplets, as previously described for the epidermis of an adult turtle (MATOLTSY & HUSZAR, 1972 ; MATOLTSY & BEDNARZ, 1975).

In the neck region, the epidermis was linear or arranged in irregular or symmetrical dermo-epidermal elevations and folds (Fig. 2). At ES 22, the epidermis appeared generally composed of 3-4 layers of cells under a flat peridermis. One to two subperidermal layers of flat cells and 1 suprabasal layer were present. The dermis, in particular near the shell openings where the neck and limbs exit, was composed of fusoid or flat fibroblasts surrounded by thick bundles of collagen fibrils (Fig. 3). The orientation of such collagen bundles was often undulated like the epidermis.

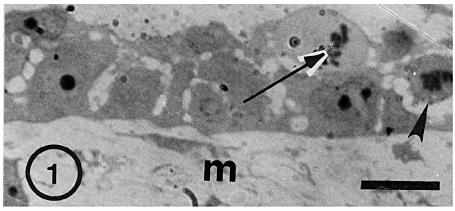
In limbs, skin foldings formed symmetric or even asymmetric scales with a longer outer side and a shorter inner side (Fig. 4). At ES 22, the whole epidermis showed the same thickness along the entire skin surface and was composed of 1-2 flattened cell layers under a superficial peridermis. Above the basal layer one suprabasal layer was present. Dermal fibroblasts and fibers followed the epidermal outline. At ES 23, there were 3-4 flattening suprabasal cell layers, both in the outer and inner sides of the scales, beneath the external peridermis, which often formed a thin, darker layer (Fig. 5). Clear droplets or vesicles were observed in the suprabasal cells.

The skin of the tail showed a more or less irregular outline, or was often folded into large dermo-epidermal bumps (Figs 6, 7). At ES 22, epidermal cells in this region were cuboidal or flat, and were irregularly stratified, with a wave-like disposition. These keratinocytes showed an irregular cellular and nuclear outline.

At ES 23, under the flat external peridermis, 3-4 suprabasal layers were seen. The cytoplasm of the more superficial and flattened suprabasal layers became dark in patches (Figs. 5).

At ES 24, the epidermis contained 2-3 suprabasal cell layers beneath 2 flat subperidermal darker layers.

At ES 25, 5-6 layers of differentiating corneocytes were visible beneath 2-4 external dark layers (Fig. 8). The peridermis and the more external layers were very flat, anucleated, dark and largely cornified.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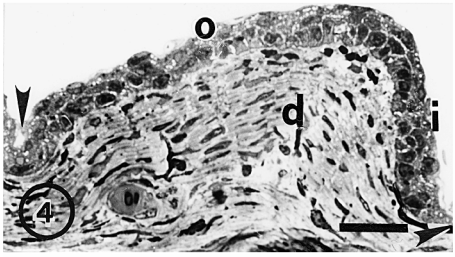
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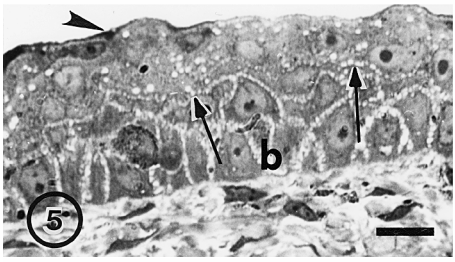
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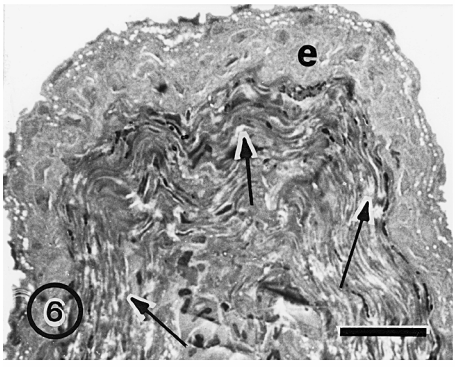
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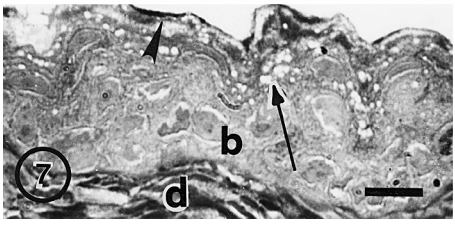
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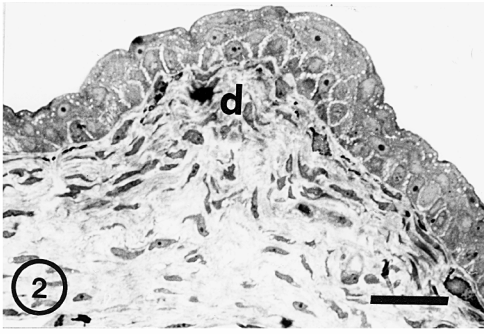
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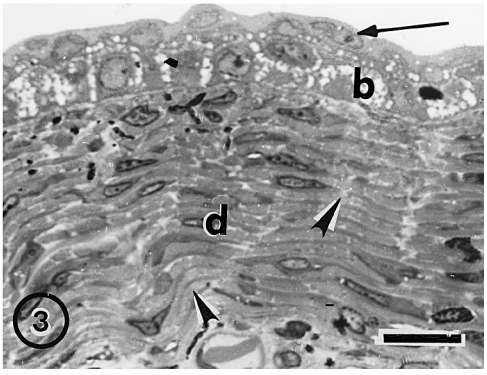
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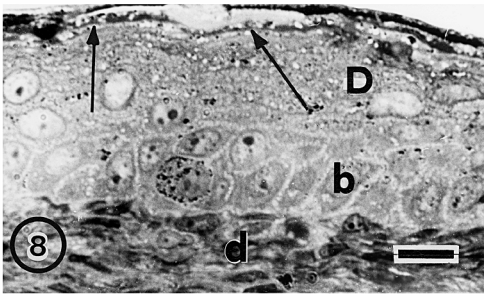
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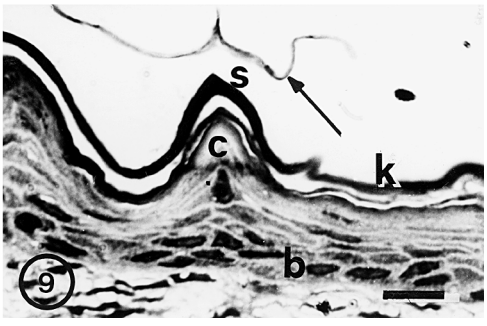
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Finally, from 2-3 days to 1 week post-hatching, the epidermis appeared covered by a dark corneous stratum, composed of narrow layers of desquamating keratinocytes (Fig. 9). The basal layer was formed by flat to cubic cells, followed by 2-4 suprabasal layers of flat cells. Above suprabasal cells the nuclei disappeared, and a 5-10 μm-thick pale layer without keratohyaline granules preceded the corneous layer. In various regions along the epidermis corneous spurs were formed.

Electron microscopy

Between ES 15 and ES 17 the cytoplasm of peridermal and epidermal cells contained most ribosomes and few, sparse, glycogen particles. Occasional isolated 8-12 nm-thick (intermediate) keratin filaments were also present in the cytoplasm of these cells but no bundles of keratin filaments were seen. The latter appeared in suprabasal cells at ES 19, and by ES 22 they were also present in the basal cells of the epidermis. A flat peridermis remained over the stratifying epidermis up to ES 24, but peridermal cells were sometimes detached or missing on the external surface. The first 3-4 layers underneath the peridermis showed peculiar cytological characteristics and, since they disappeared after ES 24, have been termed embryonic epidermal layers (ALIBARDI & THOMPSON, 1999a).

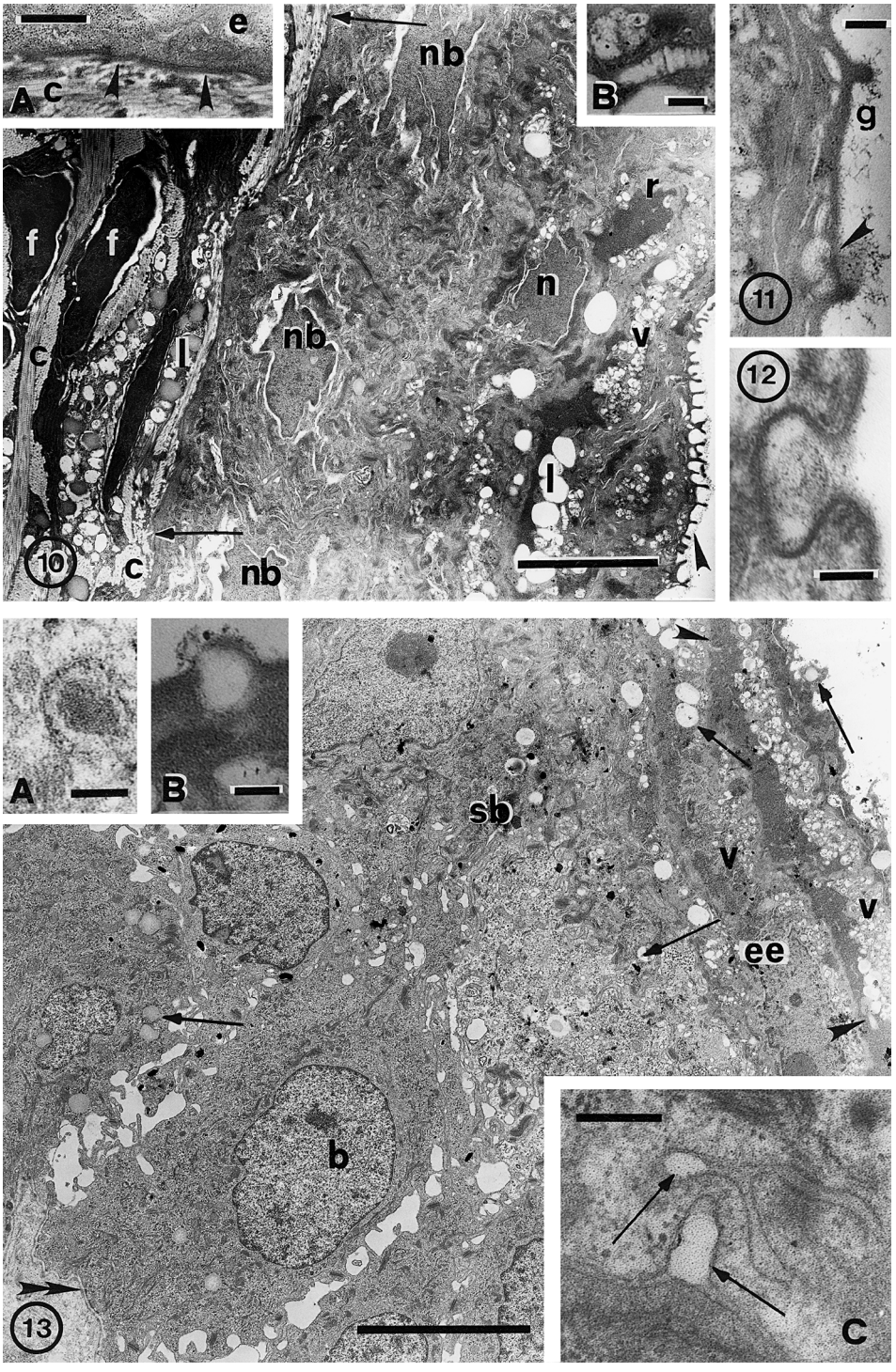
At ES 22, epidermal cells of the tail were irregular, their cell and nuclear outline was indented or irregular, forming a jig-saw puzzle-like epithelium (Figs 7, 10). As in other epidermal areas, no nuclear condensation (apoptosis) was visible in suprabasal cells up to ES 24.

The lamina densa of the basement membrane was discontinuous or lacking, and contacted by large, electron-pale collagen bundles of the dermis (Fig. 10 inset A). At ES 22, the dermis was already composed of thick criss-cross or plywood oriented collagen bundles. Lipidic material was also accumulated in some dermal cells (Fig. 10).

On the external surface of the peridermis and superficial embryonic epidermis vesicles similar to those containing mucus (PAS positive, see MATOLTSY & HUSZAR, 1972;

Legend to the figures (see page 394)

Figs 1-9. – Light microscopic observation. – 1. ES 15. Mitotic cells in the peridermis (arrow) and basal epidermis (arrowhead) of an arm. m, dermal mesenchyme. Bar=10 μm. – 2. ES 22. Epidermal bump in distal neck region, showing little epidermal stratification. The dermis (d) is composed of flat fibroblasts. Bar=20 μm. – 3. ES 22. Epidermis of proximal neck with few cells (arrow) above the basal layer (b). The dense dermis (d) shows flat fibrocytes among collagen bundles (arrowheads). Bar=20 μm. – 4. ES 22. Arm scale showing the same epidermal thickness in the outer (o) and inner (i) sides. d, oriented dermal fibroblasts. Arrowheads on hinge regions. Bar=20 μm. – 5. ES 23. Close view of leg epidermis with 3-4 suprabasal cells (arrows on pale vescicles) beneath the dark narrow peridermis (arrowhead). b, basal layer. Bar=10 μm. – 6. ES 22. Bump-like epidermal folding (e) in the tail skin with thick wavy dermis (arrows). Bar=20 μm. – 7. Close-up of tail epidermis at ES 22 showing numerous pale vesicles (arrow) localized under the dark peridermis (arrowhead). b, basal layer. Bar=10 μm. – 8. ES 25. Stratified arm epidermis of an outer scale with dark external layers (arrows). D, suprabasal differentiating cells. b, basal layer. d, thick dermis. Bar=10 μm. – 9. Digit epidermis 1 week post-hatching with a keratinized layer (k) above a pale (c) and a living flat suprabasal and basal layers (b). The embryonic epidermis (arrow) is detached. s, spurr. Bar=10 μm.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MATOLTSY & BEDNARZ, 1975) were seen fused with the external membrane, suggesting they are secretory vesicles (Figs 11, 12, 13A). Microvilli, often coated with a glycocalyx, were present in the outer peridermis (Figs 10, 11, 13, 14).

At ES 22-23, sparse bundles of intermediate filaments of keratin, and free or clumped ribosomes were visible in basal and suprabasal cells. Few ergastoplasmic cisternae, mitochondria and glycogen particles were present. In the external 3-4 epidermal layers, keratin filaments were scarce, and broad cytoplasmic areas were occupied by 28-35 nm-thick coarse filaments. The latter formed reticulate bodies that correspond to the dark patches observed in the external epidermal cells with the light microscope (Figs 5, 7, 8, 10, 13, 14, 15). Electron-pale lipid droplets (not surrounded by a membrane) and vesicular bodies (surrounded by a membrane), were sparse throughout the epidermis but were more common in the upper flat layers (Figs 10, 13, 14). Vesicular bodies were 0.2-0.4 μm-large, and contained amorphous material/membranes. These organelles were often associated with lipid droplets, with the Golgi apparatus or with the smooth endoplasmic reticulum (Figs 13 C, 16, 17). Some lipid droplets and vesicular bodies were seen on the external surface of embryonic epidermal cells, suggesting they were discharging their content onto the outer surface (Fig. 13). Other vesicular bodies containing an electron-dense material (MATOLTSY & HUSZAR, 1972 ; LANDMANN, 1986), were also present in these embryonic cells (Figs 13, 14B, 18). Some lamellation pattern was occasionally seen within the electron-dense matrix of these organelles.

At ES 24, more bundles of keratin filaments accumulated in the lower-most differentiating suprabasal cells, but no dark (apoptotic) nuclei were seen. At ES 25, beneath the first 3-4 embryonic layers, numerous bundles of keratin filaments increased in suprabasal cells and accumulated within the keratinizing cells (Fig. 19). Reticulate bodies disappeared in these forming α-keratinocytes, while vesicular bodies were reduced in number. Two layers of forming α-keratinocytes were present beneath the embryonic layers and above 2-4 layers of living suprabasal cells. These electron-dense and thin (0.1-0.5 μm-thick) keratinocytes also incorporated some melanosomes, and formed the definitive α-

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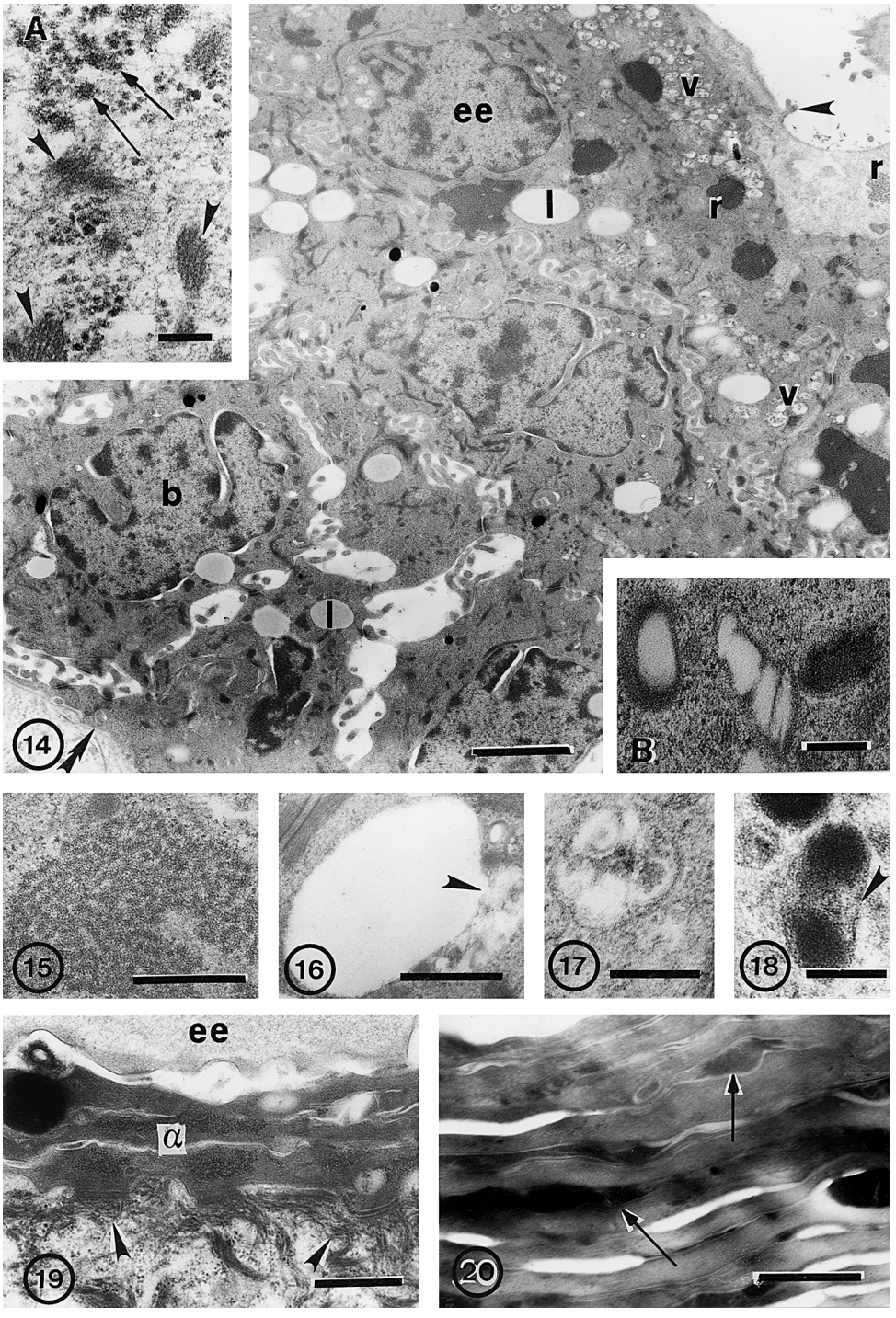
Legend to the figures (see page 396)

Figs 10-13. – Electron microscopic observations. – Electron-micrograph of tail epidermis at ES 22. Basal (nb) and suprabasal (n) nuclei are irregularly indented. Many vesicular bodies (v) and lipid droplets (l) are present in the external layers and in dermal fibroblasts (f). r, dense reticulate bodies. c, electron-pale collagen fibrils. Arrowhead on coated microvilli. Arrows point to the basement membrane. Bar=5 μm. Inset **A** illustrates the contact (arrowheads) of pale collagen fibrils (c) with the basement membrane of epidermal cells (e). Bar=1 μm. Inset **B** on two vesicular bodies. Bar=250 nm. – 11. Embryonic tail epidermis at ES 22. Discharging mucus vesicles (arrowhead) on the external epidermal surface forming a glycocalyx (g). Bar=250 nm. – 12. Embryonic tail epidermis at ES 22. Discharging or invaginating vesicle containing a low electron-dense amorphous material. Bar=100 nm. – 13. Limb epidermis at ES 23 showing suprabasal (sb) and flat embryonic epidermis (ee) layers, rich in lipid vescicles (arrows) and vesicular bodies (v). Arrowheads on dense areas occupied by reticulate bodies. b, basal cells. Double arrowhead on the basement membrane. Bar=5 μm. Inset **A**, dense-cored mucus granule. Bar=100 nm. Inset **B**, discharging lipid-like vesicle on the epidermal surface. Bar=100 nm. Inset **C**, blebbing pale vesicles (arrows) from smooth endoplasmic reticulum. Bar=200 nm.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keratin layer. No keratohyaline granules were seen in these cells. Although some nuclei showed nuclear clumping during keratinization, the nuclear modifications during keratinization were not specifically studied in this report.

More α-keratinocytes continued to accumulate in post-hatching epidermis, which consisted of 7-15 layers (depending upon the body region) of very narrow horny cells (Figs 9, 19, 20). Extracellular dense deposits of mucus or lipidic material similar to those previously described (MATOLTSY & HUSZAR, 1972 ; MATOLTSY & BEDNARZ, 1975), were present.

DISCUSSION

Epidermis

The liquid environment of the embryo of *Emydura* is in contact with a soft, non-keratinized external peridermis, which is lost, partly in ovo and completely a few days after hatching.

Beneath the peridermis, the 3-4 layers of embryonic epidermis do not cornify by accumulation of keratin bundles, as in the adult epidermis. Instead they contain mucus, vesicular bodies and lipids, which are secreted extracellularly. This secretory epithelium is visible until ES 24, and although sparse keratin bundles were present, its main cytoskeletal components are 28-35 nm-thick coarse filaments aggregated into reticulate bodies. The latter are organelles typical of peridermal cells (MOTTET & JENSEN, 1968 ; PARAKKAL & MATOLTSY, 1968 ; DHOUAILLY & MADERSON, 1984 ; SAWYER *et al.*, 1986 ; ALIBARDI, 1997, 1998a,b ; ALIBARDI & THOMPSON, 1999a), but their molecular nature is unknown. From ES 25 onward, the condensation of reticulate bodies with the scarce keratin filaments, determines the corneification and darkening of the embryonic layers. Reticulate bodies have been described in other mucus-secreting epithelia, both embryonic (SAWYER *et al.*, 1986 ; ALIBARDI, 1998a,b) and adult (FUKUYAMA & EPSTEIN, 1973).

Legend to the figures (see page 398)

Figs 14-20. – Neck epidermis at ES 23 showing accumulation of vesicular (v) and reticulate (r) bodies in external embryonic epidermal cells (ee). The arrowhead points to a microvillus. Lipidic vescicles (l) are also present in the basal layer (b). Double arrowhead on dense basement lamella. Bar=2 mm. Inset **A** on coarse filaments (arrows) of a reticulate body. Arrowheads on keratin bundles. Bar=100 nm. Inset **B** shows vesicular bodies with dense areas. Bar=250 nm. – 15. Detail of a reticulate body of embryonic epidermal cell of the neck at ES 23. Bar=500 nm. – 16. Lipid droplet contacting a vesicular body (arrowhead) in embryonic neck epidermal cell at ES 23. Bar=500 nm. – 17. High magnification of a vesicular body in embryonic epidermal cell of neck at ES 23. Bar=200 nm. – 18. Dense body surrounded by a membrane (arrowhead) in embryonic epidermal cell of neck at ES 23. Bar=250 nm. – 19. Limb scale epidermis at ES 25 showing α-keratinized cells (α) under the embryonic epidermis (ee). Arrowheads indicate aggregation of bundles of keratin filaments in differentiating α-cell. Bar=500 nm. – 20. External narrow α-keratinocytes of an arm with dense intercellular material (arrows) at 1 week post-hatching. Bar=500 nm.

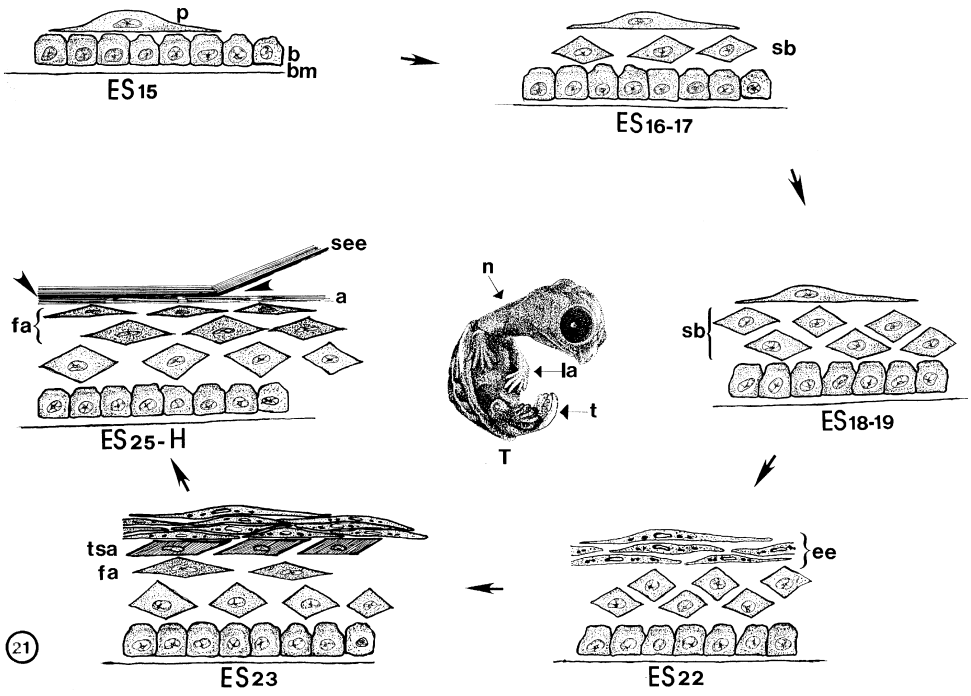

Fig. 21 – Schematic drawing of the stratification in the soft embryonic epidermis of the turtle embryo (**T**). **a**, α-keratin layer. **b**, basal layer. **bm**, basement membrane. **ee**, embryonic epidermis. **ES**, embryonic stages. **fa**, forming α-layer. **H**, hatching to about 1 week post-hatching. **la**, limb-arm. **n**, neck. **p**, periderm. **sb**, suprabasal layer. **t**, tail. **tsa**, transition α-layer between embryonic to α-keratin layer. **see**, shedding embryonic epidermis along a shedding line (**arrowheads**).

In conclusion, the embryonic epidermis shows characteristics of the mucus-secreting epidermis of amphibians (PARAKKAL & MATOLTSY, 1964; PARAKKAL & ALEXANDER, 1972; LAVKER, 1974; MATOLTSY, 1987) together with the lipid-secreting characteristics of the epidermis of aves (MATOLTSY, 1969; PARAKKAL & ALEXANDER, 1972; SAWYER & BORG, 1979; LANDMANN, 1980; SAWYER *et al.*, 1986; MENON *et al.*, 1986, 1996). Lipids, probably of polar type (MENON *et al.*, 1986), were perhaps an evolutionary addition to mucus for terrestrial life. Mucus granules with a dense core, such as those described in the adult turtle epidermis (ALEXANDER, 1970; MATOLTSY & HUSZAR, 1972), were observed less frequently in the embryonic epidermis of *Emydura*, but appeared in progressive stages of condensation (LANDMANN, 1986).

Lipid droplets in basal cells increase in number and associate with vesicular bodies. The latter resemble the lamellar bodies of adult turtle epidermis (MATOLTSY & BEDNARZ, 1975), although a regular lamellation pattern was not visible in our preparations. Since the latter organelles appeared more frequently in the more superficial embryonic layers, it seems possible that they were derived from some kind of modification of the lipid droplets present in the lower-most layers. In birds, however, lipid droplets in the intermediate to

upper epidermal layers appear to be derived from the loss of organization of lamellate bodies (MENON *et al.*, 1986 ; 1996). Vesicular bodies may also be directly produced from the smooth endoplasmic reticulum and Golgi apparatus of suprabasal cells, as in the chick epidermis (MATOLTSY, 1969 ; LAVKER, 1975), and progressively accumulate in the most superficial layers. However, the knoweldge of the precise origin of these organelles awaits more dynamic studies.

Mucus and lipids coat the epidermal surface, probably in relation to the impermeabilization of the skin to prevent inward-outward water movements in the acquatic environment.

From ES 24 onward, before the transition from the liquid to the terrestrial environment, the transformation from a mucus-lipidic-secreting epidermis to an α-keratin-producing epidermis takes place. The new epidermis is more suitable for protection against desiccation, mechanical wear, or injuries (ALEXANDER, 1970 ; HENRIKSON & MATOLTSY, 1970 ; MATOLTSY & HUSZAR, 1972). A similar epithelial transformation takes place in the dorsal lingual epithelium of freshwater turtles in contrast to more terrestrial tortoises or lizards (IWASAKI *et al*., 1996a,b), and also in the buccal epithelium of the rat (FUKUYAMA & EPSTEIN, 1973).

In the shell region, from ES 25 onward, beneath a similar embryonic epidermis a cellularized β-keratin layer is formed (ALIBARDI & THOMPSON, 1999a). The process of accumulation of β-keratin takes place with a similar modality to that described in other reptiles (ALEXANDER, 1970 ; PARAKKAL & ALEXANDER, 1972 ; LANDMANN, 1986 ; MADERSON *et al.*, 1998).

From ES 25 onward, bundles of keratin filaments increase in the newly generated α-keratinocytes, and they mix with lipids and mucus, as previously reported in the adult epidermis (MATOLTSY & HUSZAR, 1972 ; MATOLTSY, 1987). While lipids and mucus appear to decrease in quantity, reticulate bodies completely disappear in α-keratinocytes.

At 2 days post-hatching, the embryonic epidermis is reduced to a thin dark layer that is partially or totally detached from the definitive stratum corneum. At 1 week post-hatching, some 10-20 layers of α-keratinocytes are already present and the germinative epithelium becomes cuboidal or even flat (SPEARMAN, 1969 ; MATOLTSY & HUSZAR, 1972), a condition that may be related to a slowing down of the process of keratinization. In fact, in lepidosaurian reptiles the epidermal flattening that follows the loss of the old epidermis is related to the resting phase in the production of suprabasal cells (MADERSON, 1985 ; LANDMANN, 1986 ; MADERSON *et al*., 1998).

It is not known whether the soft epidermis of the neck, limbs, and tail of some Chelonia is also subject to alternating periods of high and low proliferative activity, as in the case of the epidermis of shell scutes (SPEARMAN, 1966 ; ZANGERL, 1969).

Dermis

The initial mesenchyme at ES 16 rapidly becomes a dense connective tissue, by the accumulation of thick collagen bundles among fibroblasts. At ES 22, when the epidermis is still immature, the dermis already resembles that of the adult (MATOLTSY & HUSZAR, 1972 ; LANDMANN, 1986).

The high content of collagen fibrils among oriented fibroblasts determines a reinforcement of the dermis in support of the epidermis integrity during folding of neck, limbs and tail. Dermal cells also accumulate fat.

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